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(54) Title: METHODS, COMPOSITIONS, AND CELLS FOR ENCAPSIDATING RECOMBINANT VECTORS IN AAV PARTICLES (54) Titre: PROCEDES, COMPOSITIONS ET CELLULES SERVANT A ENCAPSIDER DES VECTEURS DE RECOMBINAISON DANS DES PARTICULES DE VIRUS ADENO-ASSOCIES		
(57) Abstract Isolated recombinant polynucleotides comprising elements which promote encapsidation into AAV particles, packaging cells comprising the recombinant polynucleotides, and methods for their use are provided in the present invention. These isolated recombinant polynucleotides comprise a non-AAV ITR encapsidation element (such as the P1 sequence located within the AAV S1 integration site of human chromosome 19) operably linked to one or more heterologous genes to be encapsidated. The constructs may be either integrated into a mammalian cell genome, maintained episomally, or provided transiently.		
(57) Abrégé Polynucléotides de recombinaison isolés comprenant des éléments favorisant l'encapsidation dans des particules de virus adéno-associés (AAV), cellules de conditionnement comprenant ces polynucléotides de recombinaison et procédés permettant de les utiliser. Ces polynucléotides de recombinaison isolés comprennent un élément d'encapsidation différent d'une répétition terminale inverse (ITR) de AAV (tel que la séquence P1 située à l'intérieur du site d'intégration S1 de AAV du chromosome humain 19) relié à un ou plusieurs gènes hétérologues à encapsider. Ces produits de recombinaison peuvent être intégrés dans un génome cellulaire mammifère, maintenus dans l'épisome ou produits de façon transitoire.		

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(54) Title: METHODS, COMPOSITIONS, AND CELLS FOR ENCAPSIDATING RECOMBINANT VECTORS IN AAV PARTICLES

(57) Abstract

Isolated recombinant polynucleotides comprising elements which promote encapsidation into AAV particles, packaging cells comprising the recombinant polynucleotides, and methods for their use are provided in the present invention. These isolated recombinant polynucleotides comprise a non-AAV ITR encapsidation element (such as the P1 sequence located within the AAV S1 integration site of human chromosome 19) operably linked to one or more heterologous genes to be encapsidated. The constructs may be either integrated into a mammalian cell genome, maintained episomally, or provided transiently.

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Description

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**METHODS, COMPOSITIONS, AND CELLS FOR ENCAPSIDATING
RECOMBINANT VECTORS IN AAV PARTICLES**

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CROSS-REFERENCE TO RELATED APPLICATIONS

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This application claims priority to U.S. provisional application 60/135,119 (converted from U.S. Serial No. 09/301,514), filed April 28, 1999, and U.S. application (serial number not yet assigned) filed April 27, 2000, which are incorporated by reference in their entirety.

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STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER

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FEDERALLY SPONSORED RESEARCH

(Not applicable)

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TECHNICAL FIELD

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This invention is in the field of recombinant DNA constructs for gene delivery. More specifically, the invention is in the field of recombinant DNA constructs for use in the production of recombinant DNA vectors for gene delivery.

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BACKGROUND ART

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Vectors based on adeno-associated virus (AAV) are believed to have utility for gene therapy but a significant obstacle has been the difficulty in generating such vectors in amounts that would be clinically useful for human gene therapy applications. This is a particular problem for *in vivo* applications such as direct delivery to the lung. Another important goal in the gene therapy context, discussed in more detail herein, is the production of vector preparations that are essentially free of replication-competent virions. The following description briefly summarizes studies involving adeno-associated virus and AAV vectors, and then describes a number of novel improvements according to the present invention that are useful for efficiently generating high titer recombinant AAV vector (rAAV) preparations suitable for use in gene therapy.

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Adeno-associated virus is a defective parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. General reviews of AAV may

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5 be found in, for example, Carter, 1989, Handbook of Parvoviruses, Vol. I, pp. 169-228, and
10 Berns, 1990, Virology, pp. 1743-1764, Raven Press, (New York). Examples of co-
15 infecting viruses that provide helper functions for AAV growth and replication are
adenoviruses, herpesviruses and, in some cases, poxviruses such as vaccinia. The nature of
the helper function is not entirely known but it appears that the helper virus indirectly
renders the cell permissive for AAV replication. This belief is supported by the
observation that AAV replication may occur at low efficiency in the absence of helper virus
co-infection if the cells are treated with agents that are either genotoxic or that disrupt the
cell cycle.

20 Although AAV may replicate to a limited extent in the absence of helper virus,
under such conditions as noted above, more generally infection of cells with AAV in the
absence of helper functions results in the proviral AAV genome integrating into the host
cell genome. Unlike other viruses, such as many retroviruses, it appears that AAV
generally integrates into a unique position in the human genome. Thus, it has been
25 reported that, in human cells, AAV integrates into a unique position (referred to as an
“AAV integration site”) which is located on chromosome 19. See, e.g., Weitzman et al.
(1994) Proc. Natl. Acad. Sci. USA 91: 5808-5812. If host cells having an integrated AAV
30 are subsequently superinfected with a helper virus such as adenovirus, the integrated AAV
genome can be rescued and replicated to yield a burst of infectious progeny AAV particles.
35 A sequence at the AAV integration site, referred to as “P1”, shares limited homology with
the AAV inverted terminal repeat (ITR) sequence, exhibits Rep binding activity in a cell-
free replication system, and is believed to be involved in both the integration and rescue of
AAV. See, e.g., Weitzman et al., *id.*, Kotin et al. (1992) EMBO J. 11:5071-5078, and
40 Urcelay et al., J. Virol. 69: 2038-2046. The fact that integration of AAV appears to be
efficient and site-specific makes AAV a useful vector for introducing genes into cells for
uses such as human gene therapy.

45 AAV has a very broad host range without any obvious species or tissue specificity
and can replicate in virtually any cell line of human, simian or rodent origin provided that
an appropriate helper is present. AAV is also relatively ubiquitous and has been isolated
50 from a wide variety of animal species including most mammalian and several avian
species.

5 AAV is not associated with the cause of any disease. Nor is AAV a transforming or
oncogenic virus, and integration of AAV into the genetic material of human cells generally
does not cause significant alteration of the growth properties or morphological
characteristics of the host cells. These properties of AAV also recommend it as a
10 potentially useful human gene therapy vector because most of the other viral systems
proposed for this application, such as retroviruses, adenoviruses, herpesviruses, or
poxviruses, are disease-causing.

15 Although various serotypes of AAV are known to exist, they are all closely related
functionally, structurally, and at the genetic level (see, e.g., Blacklow, 1988, pp. 165-174
20 of Parvoviruses and Human Disease, J.R. Pattison (ed.); and Rose, 1974, Comprehensive
Virology 3: 1-61). For example, all AAV serotypes apparently exhibit very similar
replication properties mediated by homologous *rep* genes; and all bear three related capsid
25 proteins such as those expressed in AAV2. The degree of relatedness is further suggested
by heteroduplex analysis which reveals extensive cross-hybridization between serotypes
along the length of the genome; and the presence of analogous self-annealing segments at
the termini that correspond to inverted terminal repeats (ITRs). The similar infectivity
30 patterns also suggest that the replication functions in each serotype are under similar
regulatory control. Thus, although the AAV2 serotype was used in various illustrations of
the present invention that are set forth in the Examples, general reference to AAV herein
35 encompasses all AAV serotypes, and it is fully expected that the methods and
compositions disclosed herein will be applicable to all AAV serotypes.

40 AAV particles comprise a proteinaceous capsid having three capsid proteins, VP1,
VP2 and VP3, which enclose a DNA genome. The AAV2 DNA genome, for example, is a
45 linear single-stranded DNA molecule having a molecular weight of about 1.5×10^6 daltons
and a length of about 5 kb. Individual particles package only one DNA molecule strand,
but this may be either the "plus" or "minus" strand. Particles containing either strand are
50 infectious and replication occurs by conversion of the parental infecting single strand to a
duplex form and subsequent amplification of a large pool of duplex molecules from which
progeny single strands are displaced and packaged into capsids. Duplex or single-strand
copies of AAV genomes can be inserted into bacterial plasmids or phagemids and
transfected into adenovirus-infected cells; these techniques have facilitated the study of
AAV genetics and the development of AAV vectors.

5 The AAV genome, which encodes proteins mediating replication and encapsidation
of the viral DNA, is generally flanked by two copies of inverted terminal repeats (ITRs). In
10 the case of AAV2, for example, the ITRs are each 145 nucleotides in length, flanking a
unique sequence region of about 4470 nucleotides that contains two main open reading
15 frames for the *rep* and *cap* genes (Srivastava et al., 1983, *J. Virol.*, 45:555-564; Hermonat et
al., 1984, *J. Virol.* 51:329-339; Tratschin et al., 1984, *J. Virol.*, 51:611-619). The AAV2
unique region contains three transcription promoters p5, p19, and p40 (Laughlin et al.,
1979, *Proc. Natl. Acad. Sci. USA*, 76:5567-5571) that are used to express the *rep* and *cap*
genes. The ITR sequences are required in *cis* and are sufficient to provide a functional
10 origin of replication (*ori*), signals required for integration into the cell genome, and
efficient excision and rescue from host cell chromosomes or recombinant plasmids. It has
20 also been shown that the ITR can function directly as a transcription promoter in an AAV
vector. *See* Carter et al., U.S. Patent No. 5,587,308.

25 The *rep* and *cap* gene products are required in *trans* to provide functions for
replication and encapsidation of viral genome, respectively. Again, using AAV2 for
30 purposes of illustration, the *rep* gene is expressed from two promoters, p5 and p19, and
produces four proteins. Transcription from p5 yields an unspliced 4.2 kb mRNA encoding
a first Rep protein (Rep78), and a spliced 3.9 kb mRNA encoding a second Rep protein
35 (Rep68). Transcription from p19 yields an unspliced mRNA encoding a third Rep protein
(Rep52), and a spliced 3.3 kb mRNA encoding a fourth Rep protein (Rep40). Thus, the
40 four Rep proteins all comprise a common internal region sequence but differ in their amino
and carboxyl terminal regions. Only the large Rep proteins (i.e. Rep78 and Rep68) are
45 required for AAV duplex DNA replication, but the small Rep proteins (i.e. Rep52 and
Rep40) appear to be needed for progeny, single-strand DNA accumulation (Chejanovsky &
50 Carter, 1989, *Virology* 173:120-128). Rep68 and Rep78 bind specifically to the hairpin
conformation of the AAV ITR and possess several enzyme activities required for resolving
replication at the AAV termini. Rep52 and Rep40 have none of these properties. Reports
55 by C. Hölscher et al. (1994, *J. Virol.* 68:7169-7177; and 1995, *J. Virol.* 69:6880-6885) have
suggested that expression of Rep78 or Rep 68 may in some circumstances be sufficient for
infectious particle formation.

 The Rep proteins, primarily Rep78 and Rep68, also exhibit pleiotropic regulatory
activities including positive and negative regulation of AAV genes and expression from

5 some heterologous promoters, as well as inhibitory effects on cell growth (Tratschin et al.,
1986, Mol. Cell. Biol., 6:2884-2894; Labow et al., 1987, Mol. Cell. Biol., 7:1320-1325;
10 Khleif et al., 1991, Virology, 181:738-741). The AAV p5 promoter is negatively auto-
regulated by Rep78 or Rep68 (Tratschin et al., 1986). Due to the inhibitory effects of
15 expression of *rep* on cell growth, constitutive expression of *rep* in cell lines has not been
readily achieved. For example, Mendelson et al. (1988, Virology, 166:154-165) reported
very low expression of some Rep proteins in certain cell lines after stable integration of
AAV genomes.

10 The capsid proteins VP1, VP2, and VP3 share a common overlapping sequence, but
VP1 and VP2 contain additional amino terminal sequences. All three proteins are encoded
20 by the same *cap* gene reading frame typically expressed from a spliced 2.3 kb mRNA
transcribed from the p40 promoter. VP2 and VP3 can be generated from this mRNA by
use of alternate initiation codons. Generally, transcription from p40 yields a 2.6 kb
25 precursor mRNA which can be spliced at alternative sites to yield two different transcripts
of about 2.3 kb. VP2 and VP3 can be encoded by either transcript (using either of the two
initiation sites), whereas VP1 is encoded by only one of the transcripts. VP3 is the major
30 capsid protein, typically accounting for about 90% of total virion protein. VP1 is coded
from a minor mRNA using a 3' donor site that is 30 nucleotides upstream from the 3' donor
used for the major mRNA that encodes VP2 and VP3. All three proteins are required for
35 effective capsid production. Mutations which eliminate all three proteins (Cap-negative)
prevent accumulation of single-strand progeny AAV DNA, whereas mutations in the VP1
amino-terminus ("Lip-negative" or "Inf-negative") can permit assembly of single-stranded
DNA into particles but the infectious titer is greatly reduced.

40 The genetic analysis of AAV described above was largely based upon mutational
analysis of AAV genomes cloned into bacterial plasmids. In early work, molecular clones
of infectious genomes of AAV were constructed by insertion of double-strand molecules of
AAV into plasmids by procedures such as GC-tailing (Samulski et al., 1982, Proc. Natl.
45 Acad. Sci. USA, 79:2077-2081), addition of synthetic linkers containing restriction
endonuclease cleavage sites (Laughlin et al., 1983, Gene, 23:65-73) or by direct, blunt-end
30 ligation (Senapathy & Carter, 1984, J. Biol. Chem., 259:4661-4666). Transfection of such
AAV recombinant plasmids into mammalian cells that were also infected with an
appropriate helper virus, such as adenovirus, resulted in rescue and excision of the AAV

5 genome free of any plasmid sequence, replication of the rescued genome and generation of progeny infectious AAV particles. This provided the basis for performing genetic analysis of AAV as summarized above and permitted construction of AAV transducing vectors.

10 There are at least two desirable features of any AAV vector designed for use in 5 human gene therapy. The first is that the transducing vector be generated at titers sufficiently high to be practicable as a delivery system. This is especially important for 15 gene therapy strategies aimed at *in vivo* delivery of the vector. For example, it is likely that for many desirable applications of AAV vectors, such as treatment of cystic fibrosis by 20 direct *in vivo* delivery to the airway, the desired dose of transducing vector may be from 10⁸ to 10¹⁰, or, in some cases, in excess of 10¹⁰ particles. Secondly, the vector preparations 25 are preferably essentially free of wild-type AAV virus (or any replication-competent AAV). The attainment of high titers of AAV vectors has been difficult for several reasons including preferential encapsidation of wild-type AAV genomes (if they are present or generated by recombination), and the difficulty in generating sufficient complementing 30 functions such as those provided by the wild-type *rep* and *cap* genes. Useful cell lines expressing such complementing functions have been especially difficult to generate, in part because of pleiotropic inhibitory functions associated with the *rep* gene products. Thus, cell lines in which the *rep* gene is integrated and expressed may grow slowly or express *rep* at very low levels.

35 Based on genetic analyses described above, the general principles of AAV vector construction have been described. *See*, for example, Carter, 1992, Current Opinions in Biotechnology, 3:533-539; Muzyczka, 1992, Curr. Topics in Microbiol. and Immunol., 158:97-129. AAV vectors are generally constructed in AAV recombinant plasmids by 40 substituting portions of the AAV coding sequence with foreign DNA to generate a recombinant AAV (rAAV) vector or "pro-vector". It is well established in the AAV literature that, in the vector, the terminal (ITR) portions of the AAV sequence must be 45 retained intact because these regions are required in *cis* for several functions, including excision from the plasmid after transfection, replication of the vector genome and integration and rescue from a host cell genome. In some situations, providing a single ITR 50 may be sufficient to carry out the functions normally associated with two wild-type ITRs (see, e.g., Samulski et al., WO 94/13788, published 23 June 1994).

5 As described in the art, AAV ITRs generally consist of a palindromic hairpin (HP)
structure and a 20-nucleotide region, designated the D-sequence, that is not involved in the
HP formation. Wang et al. identified AAV ITR sequences required for rescue, replication
and encapsidation of the AAV genome (Wang et al., 1996, *J. Virol.* 70:1668-1677). Wang
10 et al. (1996) reported the following: (i) two HP structures and one D-sequence are
sufficient for efficient rescue and preferential replication of the AAV DNA, (ii) two HP
structures alone allow a low level rescue and replication of the AAV DNA, but rescue and
15 replication of the AAV vector DNA sequences also occur in the absence of the D-
sequences, (iii) one HP structure and two D-sequences, but not one HP structure and one
D-sequence, also allow rescue and replication of the AAV as well as the vector DNA
10 sequences, (iv) one HP structure alone or two D-sequences but not one D-sequence alone
allows replication of full length plasmid DNA but no rescue of the AAV genome occurs,
20 (v) no rescue-replication occurs in the absence of the HP structures and D-sequence, (vi) in
the absence of the D-sequences, the HP structures are insufficient for successful
25 encapsidation of the AAV genomes, and (vii) the AAV genomes containing only one ITR
structure can be packaged into biologically active virions. Thus, Wang et al. conclude that
the D-sequence plays a crucial role in the efficient rescue and selective replication and
30 encapsidation of the AAV genome. Subsequent studies published by this group suggested
that the first 10 nucleotides of the D- sequence proximal to the hairpin structure of the ITR
20 are necessary and sufficient for optimal rescue and replication of the AAV genome (Wang
et al., 1997, *J. Virol.* 71:3077-3082). Thus, this work identifies the D-sequence as required
35 for packaging of the AAV genome.

35 The vector can then be packaged into an AAV particle to generate an AAV
transducing virus by transfection of the vector into cells that are infected by an appropriate
25 helper virus such as adenovirus or herpesvirus; provided that, in order to achieve
40 replication and encapsidation of the vector genome into AAV particles, the vector must
generally be complemented for any AAV functions required *in trans*, particularly *rep* and
45 *cap*, that were deleted in construction of the vector.

45 Such AAV vectors are among a small number of recombinant virus vector systems
30 which have been shown to have utility as *in vivo* gene transfer agents (reviewed in Carter,
1992; Muzyczka, 1992) and thus are potentially of great importance for human gene
therapy. AAV vectors are capable of high-frequency transduction and expression in a

5 variety of cells including cystic fibrosis (CF) bronchial and nasal epithelial cells (see, e.g.,
Flotte et al., 1992, Am. J. Respir. Cell Mol. Biol. 7:349-356; Egan et al., 1992, Nature,
358:581-584; Flotte et al., 1993a, J. Biol. Chem. 268:3781-3790; and Flotte et al., 1993b,
Proc. Natl. Acad. Sci. USA, 90:10163-10167); human bone marrow-derived
10 erythroleukemia cells (see, e.g., Walsh et al., 1992, Proc. Natl. Acad. Sci. USA, 89:7257-
7261); as well as brain, eye and muscle cells. AAV may not require active cell division for
15 transduction and expression which would be another clear advantage over retroviruses,
especially in tissues such as the human airway epithelium where most cells are terminally
differentiated and non-dividing.

10 There is a significant need for methods that can be used to efficiently generate
recombinant vectors encapsidated in AAV particles that are essentially free of wild-type or
20 other replication-competent AAV; and a corresponding need for cell lines that can be used
to effectively generate such recombinant vectors. The present invention provides methods,
compositions, and cells for the production of high-titer, AAV particle-encapsidated,
25 recombinant vector preparations.

30 All publications and patent applications cited herein are hereby incorporated by
reference in their entirety.

SUMMARY OF THE INVENTION

30 The present invention provides compositions and methods that, when operably
linked in *cis* to a heterologous gene, promote encapsidation of the heterologous gene into
35 an AAV particle, wherein the in *cis* encapsidation function is provided by a polynucleotide
(i.e., an encapsidation element) other than an AAV ITR or preferably, other than a D-
sequence of an AAV ITR. In particular, the inventors have found that by using one or
40 more non-AAV ITR encapsidation elements in operable linkage with a heterologous gene,
and additionally providing AAV rep and cap gene products, it is possible to obtain
encapsidation of the heterologous gene in an AAV particle. As described and exemplified
herein, heterologous gene sequences in operable linkage with a non-AAV ITR
45 encapsidation element can be integrated into the chromosome of a host cell or can be
30 maintained extrachromosomally as an episome. The methods and compositions of the
present invention can be used to generate stable producer cells that are capable of
supporting production of a very large burst of AAV particles containing a recombinant

5 vector (recombinant polynucleotide), upon infection with a suitable helper virus (such as adenovirus) or provision of helper functions.

10 Accordingly, in one embodiment, the invention provides an isolated recombinant polynucleotide sequence comprising a heterologous gene operably linked to an 15 encapsidation element other than an AAV ITR or a D-sequence of an AAV ITR. In some 20 of these embodiments, the encapsidation element is a P1 element, as described herein.

25 In additional embodiments, the invention provides methods for producing high-titer stocks of recombinant vectors containing a foreign gene encapsidated in an AAV particle, by providing a mammalian cell which produces AAV rep and cap gene products and which 10 contains the recombinant vector comprising a heterologous gene operably linked to an 20 encapsidation element other than an AAV ITR or preferably, other than a D-sequence of an 25 AAV ITR.

30 The invention also provides compositions and methods for producing cell lines 20 which comprise a recombinant vector comprising a heterologous gene operably linked to an 25 encapsidation element other than an AAV ITR or a D-sequence of an AAV ITR, which 30 synthesize AAV rep and cap gene products, and which encapsidate the recombinant vector in an AAV particle; cells and cell lines produced thereby; compositions and methods for 35 high-efficiency packaging of a recombinant vector containing a heterologous gene; and recombinant vectors packaged according to the method of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

35 Figure 1 depicts a nucleotide sequence of a SmaI fragment comprising a P1 element. SmaI sites are underlined.

40 Figure 2 depicts a nucleotide sequence alignment between the nucleotide sequence 25 of a 62-nucleotide P1 encapsidation element (upper line) and nucleotides 145-79 of AAV2 ITR (lower line). The terminal resolution site is underlined, a Rep68/Rep78 binding site is indicated in bold, and the 20-nucleotide D sequence of the AAV2 ITR is italicized and in 45 bold. Vertical lines indicate nucleotide identity. A gap, indicated by dashes, of five 30 nucleotides was introduced into the P1 sequence for optimal alignment.

50 Figure 3 depicts nucleotide sequence alignments between the nucleotide sequence of a 62-nucleotide P1 encapsidation element (upper lines) and nucleotides of ITRs of various AAV serotypes (lower lines). The terminal resolution site is underlined, a

5 Rep68/Rep78 binding site is indicated in bold and vertical lines indicate nucleotide identity. As with the alignment with the AAV2 ITR shown in Fig. 2, a gap, indicated by dashes, was introduced into the P1 sequences for optimal alignment.

10 Figure 4 shows a map of the p5repCapDHFR plasmid.

5 Figure 5 shows a map of the P1RCD plasmid.

15 Figure 6 depicts an autoradiograph showing results of experiments performed to determine the sizes of recombinant vectors encapsidated in AAV particles, as described in Example 3. Numbers on the left-hand side are sizes, in kilobases, of DNA. Lane 1, 4.8 kb Bgl II to Nae I fragment from plasmid P1RCD; lane 2, 10⁸ DRP of lysate from C29 cells; lane 3, 10⁸ DRP of lysate from C29 cells treated with DNase; lane 4, 10⁹ DRP of lot 1 purified virions from P1/ACAPSN; lane 5, 10⁸ DRP of lot 1 purified virions from P1/ACAPSN; lane 6, 10⁹ DRP of lot 2 purified virions from P1/ACAPSN; lane 7, 10⁸ DRP of lot 2 purified virions from P1/ACAPSN; lane 8, 10⁸ DRP of lot 1 purified virions from P1/ALinBg; lane 9, 10⁸ DRP of lot 2 purified virions from P1/ALinBg.

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MODES FOR CARRYING OUT THE INVENTION

30 A basic challenge in the area of gene therapy is the development of strategies for efficient gene delivery to cells and tissues *in vivo*. One strategy involves the use of recombinant vectors encapsidated in AAV particles. Recombinant AAV particle-packaged 35 vectors are recombinant constructs comprising sequences required in *cis* for vector packaging, along with heterologous polynucleotide(s) encoding a protein or function of interest. Recombinant vectors packaged in AAV particles are potentially powerful tools for human gene therapy, and in general are useful for introducing a polynucleotide into a cell.

40 Although recombinant vectors packaged in AAV particles are capable of *in vivo* gene delivery, for example in the respiratory tract, high titers of such vectors are necessary to allow the delivery of a sufficiently high multiplicity of vector in a minimal volume. Consequently, optimal packaging methodology is of central importance for AAV-mediated 45 gene therapy approaches. Packaging of recombinant vectors into AAV particles is mediated in part by the products of two AAV genes: *rep* (replication proteins) and *cap* (capsid proteins), which can be provided separately in *trans*. Previously, it was believed 50 that, in addition to the *rep* and *cap* gene products provided in *trans*, an ITR was necessary

5 to provide encapsidation functions in *cis*. In addition, it was previously shown that a 20-
nucleotide portion of the AAV ITR, known as the "D sequence", plays a crucial role in the
efficient rescue and selective replication and encapsidation of the AAV genome (Wang et
al., 1996, *J. Virol.* 70:1668-1677). The inventors of this invention have made the surprising
10 5 discovery that sequences other than an AAV ITR or a D-sequence of an AAV ITR can
provide encapsidation function in *cis*.

15 The inventors of the instant invention have previously shown (in co-owned
International Patent Application No. PCT/US98/21938, the contents of which are
incorporated by reference herein) that P1 or a P1-like element provides for controlled
10 amplification of DNA comprising the P1 or P1-like element amplifiably linked to AAV rep
and cap genes, thereby providing increased template levels for synthesis of AAV packaging
20 proteins. It has now been discovered that P1 or a P1-like element can promote
encapsidation of an operably linked polynucleotide.

25 The present invention provides methods, polynucleotides, and packaging cells for
15 producing stocks of recombinant vector encapsidated in an AAV particle. A heterologous
polynucleotide is operably linked to an encapsidation element other than an AAV ITR or a
D-sequence of an AAV ITR. In some embodiments, the activating element is directly or
30 indirectly triggered by the user when it is desired to initiate vector production, preferably
by infection with helper virus or provision of helper function. The use of the P1 sequence
20 of human chromosome 19 is exemplary in these respects.

35 Without wishing to be bound by theory, it appears that upon infection or provision
of helper function, the p5 promoter is turned on to some degree, resulting in the synthesis
of some Rep protein, which may then, by acting via the encapsidation element, trigger an
encapsidation event by which the linked gene(s) are encapsidated in an AAV particle. The
25 30 encapsidation element, exemplified by P1, can thus promote encapsidation of a gene or
genes to which it is linked.

40 The invention also provides recombinant vectors comprising a heterologous gene
operably linked to an encapsidation element other than an AAV ITR or a D-sequence of an
45 30 AAV ITR. Preferably, the recombinant vector comprising the heterologous gene have a
size no greater than the upper size limit for packaging into an AAV particle, including, but
not limited to, a size of approximately 5 kb. These vectors, when encapsidated into an
AAV particle, are useful for introducing heterologous genes into a cell.

5 **General Methods**

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989); Oligonucleotide Synthesis (M.J. Gait Ed., 1984); Animal Cell Culture (R.I. Freshney, Ed., 1987); the series Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos eds. 1987); Handbook of Experimental Immunology, (D.M. Weir and C.C. Blackwell, Eds.); Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds., 1987, and updates); and Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach and W. Strober, eds., 1991).

10 All patents, patent applications, and publications mentioned herein, both *supra* and *infra*, are hereby incorporated herein by reference.

15 **Definitions**

20 As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

25 The terms "polypeptide", "peptide" and "protein" are used interchangeably to refer to polymers of amino acids of any length. These terms also include proteins that are post-30 translationally modified through reactions that include, but are not limited to, glycosylation, acetylation and phosphorylation.

35 "Polynucleotide" refers to a polymeric form of nucleotides of any length, either 40 ribonucleotides or deoxyribonucleotides, or analogs thereof. This term refers only to the primary structure of the molecule. Thus, double- and single-stranded DNA, as well as double- and single-stranded RNA are included. It also includes modified polynucleotides such as methylated or capped polynucleotides.

45 A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) 50 has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or

5 sequence identity can be determined using software programs known in the art, for
example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al.,
eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. Preferably, default parameters are
used for alignment. A preferred alignment program is BLAST, using default parameters.
10 5 In particular, preferred programs are BLASTN and BLASTP, using the following default
parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10;
Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases
15 = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations +
SwissProtein + SPupdate + PIR. Details of these programs can be found at the following
10 Internet address: <http://www.ncbi.nlm.nih.gov/BLAST>.

20 A "gene" refers to a polynucleotide containing at least one open reading frame that
is capable of encoding a particular protein after being transcribed and translated.

25 15 As used herein, "an encapsidation element other than an AAV ITR or a D sequence
of an AAV ITR", used interchangeably herein with "a packaging signal other than an
AAV ITR or a D sequence of an AAV ITR" and "a non-AAV ITR encapsidation
30 20 element", intends a polynucleotide sequence which, when operably linked in *cis* to a
heterologous gene, promotes (or enhances or increases) encapsidation of the heterologous
35 35 gene into an AAV particle, when AAV rep and cap gene products are provided in *trans*.
For the purposes of the present invention, an encapsidation element is not an AAV ITR or
a D sequence of an AAV ITR. AAV ITRs and their D sequences are known in the art, and
those skilled in the art, given the guidance provided herein, can readily determine whether
a given encapsidation element is an AAV ITR or an AAV ITR D sequence or a non-AAV
ITR encapsidation element.

40 25 As used herein, the terms "heterologous gene operably linked to an encapsidation
element", "heterologous polynucleotide operably linked to an encapsidation element",
45 30 used interchangeably herein, refer to a polynucleotide sequence which is not normally
associated in nature with a given encapsidation element.

50 35 In the context of the physical linkage between a heterologous gene and an
encapsidation element, the term "operably linked", as used herein, intends a physical
and/or functional arrangement of a heterologous gene and an encapsidation element that
permits the encapsidation element to function in *cis*, in the presence of AAV rep and cap
gene products, to encapsidate the heterologous gene in an AAV particle. Methods of

5 determining whether a given encapsidation element is "operably linked" to a given
heterologous gene are known in the art, and are described herein, and include, but are not
10 limited to, measuring the number of DNase-resistant particles (DRPs) which contain the
heterologous gene, as determined, for example, by hybridization analysis.

10 5 The term "ITR" refers to an inverted terminal repeat at either end of the AAV
15 genome. Generally, AAV ITRs are approximately 145 nucleotides long. The first 125
bases of the ITR can form a T shaped hairpin structure which is composed of two small
internal palindromes flanked by a larger palindrome (Muzyczka et al., 1992). ITRs have
been identified as being involved in AAV DNA replication and rescue, or excision, from
15 10 prokaryotic plasmids (Samulski et al., 1983, Cell 33:135-143, Samulski et al., 1987, J.
20 Virol. 61:3096-3101; Senapathy et al., 1984, J. Mol. Biol. 179:1-20; Gottlieb and
Muzyczka, 1988, Mol. Cell. Biol. 6:2513-2522).

25 15 As used herein, the term "D sequence of an AAV ITR" refers to a specific sequence
element within an AAV ITR which has been identified as playing a role in rescue, selective
30 15 replication and encapsidation of the AAV genome as described, for example, in Wang et
al., 1996 and Wang et al., 1997. The D sequence of an AAV2 ITR is illustrated in Figure 2
and, as used herein, a "D sequence of an AAV ITR" refers to the D sequence of AAV2 ITR
as well as D sequences of the ITRs of other AAV serotypes.

30 20 A "transcriptional regulatory sequence" as used herein, refers to a nucleotide
35 20 sequence that controls the transcription of a gene or coding sequence to which it is
operably linked. Transcriptional regulatory sequences of use in the present invention
generally include at least one transcriptional promoter and may also include one or more
enhancers and/or terminators of transcription.

40 25 A "promoter," as used herein, refers to a nucleotide sequence that directs the
transcription of a gene or coding sequence to which it is operably linked.

40 30 "Operably linked" refers to an arrangement of two or more components, wherein
the components so described are in a relationship permitting them to function in a
coordinated manner. By way of illustration, a transcriptional regulatory sequence or a
45 30 promoter is operably linked to a coding sequence if the transcriptional regulatory sequence
or promoter promotes transcription of the coding sequence. An operably linked
transcriptional regulatory sequence is generally joined in *cis* with the coding sequence, but
it is not necessarily directly adjacent to it.

5 "Recombinant," refers to a genetic entity distinct from that generally found in nature. As applied to a polynucleotide or gene, this means that the polynucleotide is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature.

10 5 "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a transcriptional regulatory sequence or promoter that is removed from its native coding 15 10 sequence and operably linked to a different coding sequence is a heterologous transcriptional regulatory sequence or promoter.

20 25 A "vector", as used herein, refers to a recombinant plasmid or virus that comprises a polynucleotide to be delivered into a host cell, either *in vitro* or *in vivo*. The polynucleotide to be delivered, sometimes referred to as a "target polynucleotide," 15 15 "transgene", or "gene of interest" may comprise a coding sequence of interest in gene therapy (such as a gene encoding a protein of therapeutic interest) and/or a selectable or detectable marker.

30 20 A "replicon" refers to a polynucleotide comprising an origin of replication which allows for replication of the polynucleotide in an appropriate host cell. Examples of replicons include episomes (including plasmids), as well as chromosomes (such as the nuclear or mitochondrial chromosomes).

35 25 An "origin," "replication origin," "ori-like sequence" or "ori element" is a nucleotide sequence involved in one or more aspects of initiation of DNA replication, such as, for example, binding of replication initiation factors, unwinding of the DNA duplex, 40 25 primer formation, and/or template-directed synthesis of a complementary strand. As discussed in detail herein and in the art, *ori*-like sequences can generally be found in any polynucleotide that is naturally replicated, including plasmids and viruses, as well as prokaryotic, mitochondrial and chloroplast genomes and eukaryotic chromosomes. Such *ori*-like sequences can be identified genetically (*i.e.*, replication-defective mutants, *ars* 45 30 sequences) or functionally (*i.e.*, through biochemical assay, electron microscopy, *etc.*), as is known in the art.

"Stable integration" of a polynucleotide into a cell means that the polynucleotide

5 has been integrated into a replicon that tends to be stably maintained in the cell. Although
10 episomes such as plasmids can sometimes be maintained for many generations, genetic
material carried episomally is generally more susceptible to loss than chromosomally-
15 integrated material. However, maintenance of a polynucleotide can often be effected by
20 incorporating a selectable marker into or adjacent to a polynucleotide, and then
25 maintaining cells carrying the polynucleotide under selective pressure. In some cases,
30 sequences cannot be effectively maintained stably unless they have become integrated into
a chromosome; and, therefore, selection for retention of a sequence comprising a
35 selectable marker can result in the selection of cells in which the marker has become
40 stably-integrated into a chromosome. Antibiotic resistance genes can be conveniently
45 employed as such selectable markers, as is well known in the art. Typically, stably-
50 integrated polynucleotides would be expected to be maintained on average for at least
about twenty generations, preferably at least about one hundred generations, still more
preferably they would be maintained permanently. The chromatin structure of eukaryotic
55 chromosomes can also influence the level of expression of an integrated polynucleotide.
Having the genes carried on stably-maintained episomes can be particularly useful where
60 it is desired to have multiple stably-maintained copies of a particular gene. The selection
65 of stable cell lines having properties that are particularly desirable in the context of the
70 present invention are described and illustrated below.

75 "AAV" is adeno-associated virus. Adeno-associated virus is a defective
80 parvovirus that grows only in cells in which certain functions are provided by a co-
85 infecting helper virus. General reviews of AAV may be found in, for example, Carter,
90 1989, Handbook of Parvoviruses, Vol. I, pp. 169-228, and Berns, 1990, Virology, pp.
95 1743-1764, Raven Press, (New York). The AAV2 serotype was used in some of the
100 illustrations of the present invention that are set forth in the Examples. However, it is
105 fully expected that these same principles will be applicable to other AAV serotypes since
110 it is now known that the various serotypes are quite closely related - both functionally and
115 structurally, even at the genetic level (see, e.g., Blacklow, 1988, pp. 165-174 of
120 Parvoviruses and Human Disease, J.R. Pattison (ed.); and Rose, 1974, Comprehensive
125 Virology 3: 1-61). For example, all AAV serotypes apparently exhibit very similar
130 replication properties mediated by homologous *rep* genes; and all bear three related capsid
135 proteins such as those expressed in AAV2. The degree of relatedness is further suggested

5 by heteroduplex analysis which reveals extensive cross-hybridization between serotypes
10 along the length of the genome; and the presence of analogous self-annealing segments at
the termini that correspond to inverted terminal repeats (ITRs). The similar infectivity
patterns also suggest that the replication functions in each serotype are under similar
5 regulatory control.

15 A "recombinant AAV vector" (or "rAAV vector") refers to a vector comprising
one or more polynucleotide sequences of interest, genes of interest or "transgenes" that are
flanked by AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be
replicated and packaged into infectious viral particles when present in a host cell that has
10 been infected with a suitable helper virus and that is expressing AAV *rep* and *cap* gene
products (i.e. AAV Rep and Cap proteins). When an rAAV vector is incorporated into a
20 larger polynucleotide (e.g. in a chromosome or in another vector such as a plasmid used
for cloning or transfection), then the rAAV vector is typically referred to as a "pro-vector"
which can be "rescued" by replication and encapsidation in the presence of AAV
25 packaging functions and necessary helper functions.

As used herein, a recombinant vector to be packaged (encapsidated) in an AAV particle intends a vector comprising one or more heterologous polynucleotide sequences, heterologous genes or “transgenes” that are operably linked to an encapsidation element other than an AAV ITR or a D-sequence of an AAV ITR. Such recombinant vectors can be replicated and packaged into infectious AAV particles when present in a host cell that has been infected with a suitable helper virus (or provided with helper function(s)) and that synthesizes AAV *rep* and *cap* gene products (i.e. AAV Rep and Cap proteins).

A "helper virus" for AAV refers to a virus that allows AAV (which is a "defective" parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses and poxviruses such as 40 vaccinia. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and available from 45 depositories such as the ATCC. Viruses of the herpes family include, for example, herpes simplex viruses (HSV) and Epstein-Barr viruses (EBV), as well as cytomegaloviruses (CMV) and pseudorabies viruses (PRV); which are also available from depositories such 30 as ATCC. "Helper function" refers to the activity provided by the helper virus that allows

5 replication and packaging of an AAV genome, or any equivalent activity. Helper
functions are also believed to stimulate transcription of some AAV promoters, including
p5, and may enhance processivity of replication in cells in which helper functions are
expressed.

10 5 "Packaging" as used herein refers to a series of subcellular events that results in the
assembly and encapsidation of all or part of a recombinant vector comprising one or more
encapsidation elements other than an AAV ITR or and AAV ITR D-sequence. Thus,
15 when a recombinant vector comprising an encapsidation element other than an AAV ITR
or its D sequence, is introduced into a packaging cell, or packaging cell line, under
10 appropriate conditions, it can be assembled into a viral particle. Functions associated with
packaging of viral vectors, particularly AAV vectors, are described herein and in the art.

20 20 AAV "*rep*" and "*cap*" genes are genes encoding replication and encapsidation
proteins, respectively. AAV *rep* and *cap* genes have been found in all AAV serotypes
examined, and are described herein and in the references cited. In wild-type AAV, the *rep*
25 15 and *cap* genes are generally found adjacent to each other in the viral genome (i.e. they are
"coupled" together as adjoining or overlapping transcriptional units), and they are
generally conserved among AAV serotypes. AAV *rep* and *cap* genes are also individually
30 30 and collectively referred to herein as "AAV packaging genes." AAV packaging genes that
have been modified by deletion or point mutation, or which have been subdivided into
20 20 components which can be rejoined by recombination (e.g., as described in co-owned
International Patent Application No. PCT/US97/23247, the disclosure of which is hereby
incorporated by reference), may also be used in the present invention. AAV packaging
35 35 genes can also be operably linked to other transcriptional regulatory sequences, including
promoters, enhancers and polyadenylation ("polyA") sequences (which additional
25 25 transcriptional regulatory sequences can also be heterologous). An "AAV packaging
cassette" is a recombinant construct which includes one or more AAV packaging genes.

40 40 "Efficiency" when used in describing a cell line refers to certain useful attributes of
the line; in particular, the growth rate, and (for packaging cell lines) the number of virus
45 45 particles produced per cell. "Efficient growth" of a packaging cell line refers to the
30 30 effective growth rate of the packaging cell, related to a comparable parental-type cell (i.e.,
a cell that does not carry an introduced AAV packaging gene). Preferably, the relative
growth rate is at least 20% of the parental type, more preferably, 40%, more preferably,

5 80%, still more preferably, 90% and, most preferably, 100%. “High efficiency packaging” indicates production of at least about 100 viral particles per cell, more preferably at least about 1,000 viral particles per cell, still more preferably at least about 10,000 viral particles per cell. “High safety packaging” indicates that, of the recombinant AAV viral
10 15 5 particles produced, fewer than about 1 in 10^6 are replication-competent AAV viral particles, preferably fewer than about 1 in 10^8 are replication-competent, more preferably fewer than about 1 in 10^{10} are replication-competent, still more preferably fewer than about 1 in 10^{12} are replication-competent, most preferably none are replication-competent. Preferred packaging cells of the present invention exhibit combinations of such high
10 efficiency and high safety.

20 "Host cells", "cell lines", "cell cultures", "packaging cell line" and other such terms denote higher eukaryotic cells, preferably mammalian cells, most preferably human cells, useful in the present invention. These cells can be used as recipients for recombinant vectors, viruses or other transfer polynucleotides, and include the progeny of the original cell that was transduced. It is understood that the progeny of a single cell may not necessarily be completely identical (in morphology or in genomic complement) to the original parent cell.

25 15

30 A “therapeutic gene”, “target polynucleotide”, “transgene”, “gene of interest”,
35 “heterologous gene”, “heterologous polynucleotide” and the like generally refer to a gene
40 or genes to be transferred using a vector. Typically, in the context of the present
45 invention, such genes are located within a recombinant vector (which vector comprises the
50 heterologous gene and one or more encapsidation elements other than an AAV ITR or its
55 D sequence) for packaging in an AAV particle. Target polynucleotides can be used in this
60 invention to generate recombinant vectors for a number of different applications. Such
65 polynucleotides include, but are not limited to: (i) polynucleotides encoding proteins
70 useful in other forms of gene therapy to relieve deficiencies caused by missing, defective
75 or sub-optimal levels of a structural protein or enzyme; (ii) polynucleotides that are
80 transcribed into anti-sense molecules; (iii) polynucleotides that are transcribed into decoys
85 that bind transcription or translation factors; (iv) polynucleotides that encode cellular
90 modulators such as cytokines; (v) polynucleotides that can make recipient cells
95 susceptible to specific drugs, such as the herpes virus thymidine kinase gene; (vi)
100 polynucleotides for cancer therapy, such as E1A tumor suppressor genes or p53 tumor

5 suppressor genes for the treatment of various cancers, (vii) polynucleotides that encode
10 antigens or antibodies and (viii) polynucleotides that encode viral proteins, including, but
not limited to, AAV Rep and Cap proteins. To effect expression of the transgene in a
15 recipient host cell, it is preferably operably linked to a promoter or other such
transcriptional regulatory sequence, either its own or a heterologous promoter. A large
number of suitable promoters are known in the art, the choice of which depends on the
desired level of expression of the target polynucleotide; whether one wants constitutive
20 expression, inducible expression, cell-specific or tissue-specific expression, etc. The
recombinant vector may also contain a selectable marker.

25 10 An "activating element" is a sequence that responds to the presence of an activation
signal by amplifying (*i.e.*, replicating the sequences) to which it is amplifiably linked. A
preferred activating element is the P1 element and preferred activation signals include
AAV helper functions (as exemplified by adenovirus E1A function) or their equivalents.
As used herein, two sequences, one of which is an activating element, are "amplifiably
30 15 linked" when they are in sufficient proximity to each other that replication initiating from
the activating element results in amplification (*i.e.*, increased copy number) of the other
sequence. Preferably, the copy number of the amplified sequence is amplified 2-fold or
greater, more preferably, 10-fold or greater, still more preferably, 20-fold or greater. It is to
be noted that the ability of an activating element to amplify an amplifiably-linked sequence
35 20 will be influenced by the degree of processivity of replication initiating from the activating
element. Thus, factors that enhance processivity of replication will tend to increase the
effective level of amplification of a sequence that is amplifiably linked to an activating
element. In the context of the present invention, infection with adenovirus, or provision of
equivalent helper function, may enhance processivity of replication as well as initiating
25 35 amplification.

40 40 **Encapsidation elements for use in recombinant vectors, packaging cells, and methods
of the invention**

45 45 The present inventors have discovered that non-AAV ITR encapsidation elements
such as the P1 sequence (normally found on human chromosome 19), when operably linked
30 50 to one or more heterologous genes, in a mammalian cell which synthesizes AAV rep and
cap gene products, can promote encapsidation of the linked heterologous gene into an AAV
particle. In particular, when a recombinant vector of the present invention comprising an

5 encapsidation element operably linked to a heterologous gene is provided in a mammalian
cell which synthesizes AAV rep and cap gene products, under suitable conditions,
including the provision of helper virus or helper function, high titers of AAV particles
containing the recombinant vector are produced by the host cells. P1 exemplifies a class of
10 5 encapsidation elements possessing, among other properties, activatable encapsidation
function, that is useful in the generating recombinant vectors encapsidated in an AAV
particle.

15 The methods and compositions of the invention will therefore utilize recombinant
DNA constructs wherein a heterologous gene is operably linked to one or more
10 10 encapsidation elements. The presently preferred encapsidation elements are exemplified by
P1 and P1-like elements that exhibit functional properties related to encapsidation functions
20 normally associated with AAV ITRs. Most preferred are elements that act as helper
function-inducible encapsidation elements.

25 The P1 element contains at least two distinct sequence motifs, a site at which Rep
15 proteins can bind, known as the "Rep-binding motif" (or "Rep-binding site" or "RB site")
and a terminal resolution site ("trs"), at which bound Rep protein can nick the DNA (see
Figure 2). During AAV replication, it is believed that Rep protein binds within the AAV
30 inverted terminal repeat and catalyzes the formation of a nick (at the terminal resolution
site), resulting in covalent attachment of Rep protein to the newly generated 5' end. The 3'
20 end of the nick serves as a primer for AAV DNA synthesis. Subsequently, operably linked
polynucleotides are encapsidated unidirectionally. Further, as shown in Example 3, either
35 of both strands of a double-stranded polynucleotide can be encapsidated. In the Examples,
encapsidation of a given polynucleotide into AAV particles is determined by measuring
DNase-resistant particles, and further by determining the polynucleotide contents of the
25 DRPs by hybridization with a labelled probe complementary to the polynucleotide. These
40 methods can be used as an assay to identify additional encapsidation elements.

45 Weitzman et al. ((1994) *Proc. Natl. Acad. Sci. USA* 91:5808-5812) reported that a
109-base pair SmaI fragment (Figure 1), designated P1, at the site of AAV integration into
the human genome specifically binds Rep 68 and Rep78 proteins. A P1 element for use in
30 the present invention can comprise this 109-bp fragment. However, as discussed below,
portions of this 109-bp fragment can function to encapsidate an operably linked
polynucleotide. In addition, longer fragments from the AAV integration site which

5 comprise this P1 element can also be used. Further, variants of this sequence can be used
to promote encapsidation of an operably linked polynucleotide sequence.

10 As shown in Figure 2, a 62-nucleotide encapsidation element, which is a sub-
fragment of the 109-bp P1 element described above, shares about 47% nucleotide sequence
15 identity when aligned with an AAV2 ITR from nucleotide 145 to 79 (Muzyczka, 1992),
where a 5-nucleotide gap is introduced between nucleotides 32 and 33 of the P1 element
20 shown in Figure 2.

25 As was done with the AAV2 ITR sequence, ITR sequences from other AAV
serotypes have also been aligned with the 62-nucleotide P1 encapsidation element (Figure
30 3). AAV ITR sequences were taken from Xiao et al., 1999, J. Virol. 73:3994-4003;
35 Muramatsu et al., 1996, Virology 221:208-217; Chiorini et al., 1997, J. Virol. 71:6823-
40 6833 and Chiorini et al., 1999, J. Virol. 73:4293-4298. As depicted in Figure 3, the P1
45 element shares about 42% nucleotide sequence identity when aligned with an AAV1 ITR,
50 the P1 element shares about 44% nucleotide sequence identity when aligned with an AAV3
55 ITR, the P1 element shares about 45% nucleotide sequence identity when aligned with an
AAV4 ITR, the P1 element shares about 53% nucleotide sequence identity when aligned
with an AAV5 ITR and the P1 element shares about 39% nucleotide sequence identity
when aligned with an AAV6 ITR.

55 In some embodiments, a non-AAV ITR encapsidation element shares at least about
60 25 to about 30%, more preferably at least about 30 to about 40%, more preferably at least
65 about 40 to about 45%, more preferably at least about 45 to about 47%, more preferably at
70 47 to about 53%, more preferably from at least about 53 to about 60%, more
75 preferably at least about 60% to about 70%, more preferably at least about 70% to about
80%, more preferably at least about 80% to about 90%, even more preferably at least about
85 90% or more sequence identity with the 62-nucleotide P1 element shown in Figure 2. In
90 some embodiments, recombinant vectors of the invention comprise one or more P1
95 elements, one or both of which have the sequence of the P1 element shown in Figure 2.

100 In some embodiments, a non-AAV ITR encapsidation element comprises a binding
site for AAV Rep68/Rep78 proteins. In some of these embodiments, the Rep68/Rep78
105 binding site has the nucleotide sequence 5' GCXCGCTCGCTGCTX, where X is any
110 nucleotide. In other embodiments, a non-AAV ITR encapsidation element comprises a
115 terminal resolution site. In some of these embodiments, a terminal resolution site has the

5 nucleotide sequence GGTTGG. In other embodiments, a non-AAV ITR encapsidation
element comprises both a Rep68/Rep78 binding site and a terminal resolution site. In some
of these embodiments, a non-AAV ITR comprises the nucleotide sequence
10 GGTTGG(X)_nGCXCGCTCGCTCGCTX, wherein X is any nucleotide and n is a number
5 from 1 to about 100, preferably about 50, more preferably about 20, more preferably about
10.

15 A non-AAV ITR encapsidation element for use in the present invention promotes
(or increases, or enhances) encapsidation of an operably linked heterologous gene into an
AAV particle. Those skilled in the art can readily determine whether a given nucleotide
10 sequence functions as an encapsidation element. Any of a variety of methods known to
those skilled in the art can be employed for this determination, including, but not limited to,
20 measuring the number of DRPs (i.e., encapsidated recombinant vectors), and subjecting the
DRPs to hybridization analysis, as described in Example 2. A non-AAV ITR
25 encapsidation element for use in the present invention promotes encapsidation of an
operably linked heterologous gene such that at least about 10², more preferably at least
about 10⁴, more preferably at least about 10⁶, more preferably at least about 10⁷, more
preferably at least about 10⁸, more preferably at least about 10⁹, even more preferably at
30 least about 10¹⁰ or more, DRP containing the heterologous gene per milliliter are generated
when the vector is provided in a mammalian cell which synthesizes AAV rep and cap gene
20 products, and to which mammalian cell is provided helper virus function(s).

35 **Isolated recombinant polynucleotides comprising a heterologous gene operably linked
to a non-AAV ITR encapsidation element**

40 Urcelay et al. ((1995) *J. Virol.* 69:2038-2046) describe a plasmid, pMAT50, which
25 comprises a P1 element and a lacZ gene and an AAV ITR. No encapsidation function was
attributed to this P1 element. The present invention provides an isolated recombinant
45 polynucleotide (also referred to herein as an isolated recombinant vector) comprising a
non-AAV ITR encapsidation element operably linked to a heterologous gene(s), wherein
30 the encapsidation element promotes encapsidation of the operably linked heterologous gene
into an AAV particle under conditions permissive for encapsidation, and wherein the
isolated recombinant vector is not pMAT50. Conditions permissive for encapsidation are
provided when the isolated recombinant polynucleotide is in a mammalian cell which

5 synthesizes AAV rep and cap gene products, and which is provided with helper virus
function. In these embodiments, the isolated recombinant polynucleotide is introduced into
a mammalian cell which synthesizes AAV rep and cap gene products. When helper virus
function is further provided, the isolated recombinant polynucleotide is encapsidated in
10 5 AAV particles.

We have observed that placing an encapsidation element, as exemplified by a P1
sequence, near a heterologous gene, e.g., a cap gene, resulted in an efficient packaging of the
15 heterologous gene in AAV particles. Indeed, as shown below, a P1 element placed at a
distance of 5.2 kb from the DHFR sequence, for example, resulted in efficient packaging of
10 the heterologous gene with production of approximately 10^{10} DRPs per milliliter. This
compares favorably with encapsidation efficiencies reported for ITR-mediated packaging
20 of AAV vector genomes. Although placing an encapsidation element further away from an
AAV packaging gene (e.g. 5-10 kb or further) may result in somewhat lower levels of
25 encapsidation, longer distances between an encapsidation element and an operably linked
heterologous gene would still be expected to provide a degree of encapsidation sufficient
15 for production of isolated recombinant polynucleotides encapsidated in AAV particles.
Accordingly, in some embodiments, the non-AAV ITR encapsidation element is less than
30 about 10 kb, more preferably less than about 5 kb, more preferably less than about 4 kb,
more preferably less than about 3 kb, more preferably less than about 2 kb, more preferably
20 less than about 1 kb, more preferably less than about 0.5 kb away from (i.e., in the direction
of encapsidation from) the isolated recombinant polynucleotide comprising a heterologous
35 gene to be packaged into an AAV particle.

In some embodiments, the isolated recombinant polynucleotide further comprises a
selectable marker. Once this recombinant polynucleotide is introduced into a mammalian
25 cell, the cell can be subjected to selection appropriate to the selectable marker. A variety of
40 selectable markers suitable for use in mammalian cells, and the manner of selection, are
known in the art, and need not be described in detail herein. Any such selectable marker is
suitable for use in the isolated recombinant polynucleotides of the invention. Mammalian
45 cells comprising an isolated recombinant polynucleotide containing a selectable marker,
30 subjected to selection appropriate to the selectable marker can yield cells which comprise
the recombinant polynucleotide stably integrated into the genome of the cell, as described
in the Examples. When such a cell synthesizes AAV rep and cap gene products, and

5 exhibits helper virus function, or is provided with helper virus function, the recombinant polynucleotide can be rescued and encapsidated into AAV particles.

10 In encapsidating copies of integrated operably linked heterologous gene(s) in response to helper virus infection, the P1 element appears to direct encapsidation 5 unidirectionally. Without wishing to be bound by theory, it is believed that interaction of Rep with a Rep-binding motif may be followed by nicking between the two T residues in a Terminal Resolution Site (TRS), as illustrated below. Subsequently, replication may 15 initiate from the 3' hydroxyl end of the nick and proceed toward the Rep-binding motif. Accordingly, in some embodiments, a unidirectional encapsidation element (for example, 20 P1) is oriented such that unidirectional replication proceeds from the encapsidation element toward the associated (i.e., operably linked) heterologous gene(s).

25 Alternatively, a heterologous gene(s) can be flanked by encapsidation elements that are oriented so that replication initiated at each element proceeds "inward" toward the 30 heterologous gene(s).

30 15 It is understood that while the polynucleotides containing the encapsidation element(s) and the heterologous gene(s) may be integrated, they may also exist in an episomal state.

35 20 In some embodiments, the isolated recombinant polynucleotides of the invention have a size no greater than the upper size limit for packaging into an AAV particle. In 25 some of these embodiments, isolated recombinant polynucleotides of the invention have a size greater than about 5 kb. In some of these embodiments, isolated recombinant 35 polynucleotides of the invention have a size less than about 5 kb. In some of these 40 embodiments, the size of the isolated recombinant polynucleotide is about 4.7 kb or less. Examples of recombinant polynucleotides sizes packageable into an AAV particle include, 45 but are not limited to, those sizes exemplified in Dong et al., 1996, Human Gene Ther. 25

Production of AAV particles comprising a heterologous gene

45 To generate recombinant AAV particles useful for such purposes as gene therapy, or introducing a transgene into a cell, a packaging cell, or a packaging cell line, which 50 synthesizes AAV rep and cap gene products, is generally supplied with a recombinant vector comprising a heterologous gene operably linked to an encapsidation element other than an AAV ITR or a D-sequence of an AAV ITR, such that the recombinant vector enters

5 the cell and is packaged into an AAV particle in the presence of helper virus function(s).
10 The vector can be introduced into the packaging cell by any known means, including, but
not limited to, electroporation and lipofection. The packaging cell provides AAV rep and
cap functions, which can be encoded by polynucleotide sequences which are stably
15 integrated into the genome, or which are maintained in the packaging cell episomally, or
are produced by transiently transfecting the cell with a vector, such as a plasmid vector,
which comprises sequences encoding AAV rep and cap gene products. Helper functions
20 can be provided by infecting the packaging cell with helper virus before, during, or after
providing the cell with the recombinant vector. Alternatively, a vector which comprises
25 nucleotide sequences which encode helper virus function(s) can be provided to the cell
before, during, or after providing the cell with the recombinant vector. In some
embodiments, the recombinant vector is provided to the cell transiently. In other
30 embodiments, the recombinant vector comprises a selectable marker and the packaging cell
is selected on the basis of the selectable marker such that the recombinant vector is stably
35 integrated into the genome of the packaging cell. In other embodiments, the recombinant
vector can stably integrate into the genome of the packaging cell without the need for a
selectable marker.

30 **Heterologous polynucleotides**

20 The heterologous polynucleotide, if it is intended to be expressed, is generally
operably linked to a promoter, either its own or a heterologous promoter. A large number
35 of suitable promoters are known in the art, the choice of which depends on the desired level
of expression of the target polynucleotide; whether one wants constitutive expression,
inducible expression, cell-specific or tissue-specific expression, etc. The recombinant
40 vector can also contain a positive selectable marker in order to allow for selection of cells
that have been infected by the recombinant vector; and/or a negative selectable marker (as a
means of selecting against those same cells should that become necessary or desirable); see,
e.g., S.D. Lupton, PCT/US91/08442 and PCT/US94/05601.

45 As an example, a recombinant vector can be constructed which comprises an
encapsidation element operably linked to a polynucleotide that encodes a functional cystic
50 fibrosis transmembrane conductance regulator polypeptide (CFTR) operably linked to a
promoter. As is now known in the art, there are a variety of CFTR polypeptides that are
capable of reconstituting CFTR activity in cells derived from cystic fibrosis patients. For
55

5 example, Carter et al. have described truncated variants of CFTR genes that encode
functional CFTR proteins (see, e.g., U.S. Patent No. 5,866,696). See also, Rich et al.
10 (1991, Science 253: 205-207) who have described a CFTR derivative missing amino acid
residues 708-835, that was capable of transporting chloride and capable of correcting a
15 naturally occurring CFTR defect, and Egan et al. (1993) who described another CFTR
derivative (comprising about 25 amino acids from an unrelated protein followed by the
sequence of native CFTR beginning at residue 119) that was also capable of restoring
electrophysiological characteristics of normal CFTR. To take two additional examples,
15 Arispe et al. (1992, Proc. Natl. Acad. Sci. USA 89: 1539-1543) showed that a CFTR
fragment comprising residues 433-586 was sufficient to reconstitute a correct chloride
20 channel in lipid bilayers; and Sheppard et al. (1994, Cell 76:1091-1098) showed that a
CFTR polypeptide truncated at residue 836 to about half its length was still capable of
building a regulated chloride channel. Thus, the native CFTR protein, and mutants and
fragments thereof, all constitute CFTR polypeptides that are useful in the practice of this
25 invention.

Other useful target polynucleotides can be used in this invention to generate
recombinant vectors for a number of different applications. Such polynucleotides include,
30 but are not limited to: (i) polynucleotides encoding proteins useful in other forms of gene
therapy to relieve deficiencies caused by missing, defective or sub-optimal levels of a
structural protein or enzyme; (ii) polynucleotides that are transcribed into anti-sense
35 molecules; (iii) polynucleotides that are transcribed into decoys that bind transcription or
translation factors; (iv) polynucleotides that encode cellular modulators such as cytokines;
(v) polynucleotides that can make recipient cells susceptible to specific drugs, such as the
40 herpes virus thymidine kinase gene; and (vi) polynucleotides for cancer therapy, such as the
wild-type p53 tumor suppressor cDNA for replacement of the missing or damaged p53
gene associated with over 50% of human cancers, including those of the lung, breast,
45 prostate and colon.

Mammalian packaging cells

45 The present invention provides mammalian packaging cells for producing stocks of
30 a recombinant polynucleotide encapsidated in an AAV particle, wherein the recombinant
polynucleotide comprises a heterologous gene operably linked to a non-AAV ITR

5 encapsidation element which promotes encapsidation of the operably linked heterologous gene into the AAV particle.

10 For production of a recombinant polynucleotide encapsidated in an AAV particle, wherein the recombinant polynucleotide comprises a heterologous gene operably linked to a non-AAV ITR encapsidation element, and preferably to a non-AAV ITR D-sequence 15 encapsidation element, a mammalian cell which synthesizes AAV rep and cap gene products, i.e., a packaging cell, is used. AAV rep and cap gene products can be encoded by stably integrated AAV rep and cap genes, or can be encoded by polynucleotides comprised in a vector which is introduced into the cell before, during, or after introduction of the 20 recombinant vector. Further, stable cell lines can be generated which comprise the recombinant vector stably integrated into the genome of the cell.

25 Since the therapeutic specificity of the resulting recombinant vector is determined by the plasmid introduced, the same packaging cell line can be used for any of these 30 applications. The plasmid comprising the specific target polynucleotide is introduced into 15 the packaging cell for production of the AAV vector by any known method; including, but not limited to, electroporation.

35 A number of packaging cells comprising stably integrated AAV cap and/or rep 40 genes are known in the art and can be used for packaging the recombinant vectors described herein. see, e.g., T. Flotte et al., WO 95/13365 (Targeted Genetics Corporation 20 and Johns Hopkins University), and corresponding U.S. Patent 5,658,776; J. Trempe et al., WO 95/13392 (Medical College of Ohio), and corresponding U.S. Patent No. 5,837,484; 45 and J. Allen, WO 96/17947 (Targeted Genetics Corporation).

50 Such packaging cells include, but are not limited to, packaging cells which 45 comprise a stably integrated AAV cap gene operably linked to a promoter and a stably 30 integrated AAV rep gene operably linked to a heterologous promoter, for example as described by Allen (International Patent Application No. PCT/US95/15892); packaging cells comprising an AAV rep gene, which may be operably linked to a heterologous promoter; packaging cells comprising an AAV cap gene operably linked to a promoter. When packaging cells comprising stably integrated rep and cap genes are used, the 55 recombinant vector comprising a heterologous gene operably linked to an encapsidation element is introduced into the cell, and, in the presence of helper virus function, the recombinant vector is packaged into AAV particles. When packaging cells comprising

5 stably integrated AAV rep or AAV cap genes are used, the missing in *trans* product is supplied, typically on a plasmid vector which is introduced before, simultaneously with, or after, introduction of the recombinant vector.

10 5 In other embodiments, the packaging cells are provided with both AAV rep and AAV cap gene products by introducing into the cell a vector comprising coding sequences for AAV rep and cap gene products before, simultaneously with, or after, introduction of the recombinant vector comprising a heterologous gene operably linked to an encapsidation element. Plasmid-encoded AAV rep and/or cap genes can optionally be maintained episomally.

15 10 In other embodiments, also illustrated in the Examples below, the recombinant vector is itself stably integrated into a packaging cell line. Such stable, vector-containing packaging lines can also optionally contain stable chromosomal or episomal copies of AAV cap and/or rep genes. Cell lines such as those described above can be grown and stored until ready for use. To induce production of recombinant vector packaged into AAV 20 15 particles in cells that contain Rep and Cap proteins, the user simply infects the cells with helper virus, or provides helper functions on a plasmid introduced by any known method, and cultures the cells under conditions suitable for replication and packaging of AAV (as described below).

25 30 **Helper virus function**
20 25 Helper virus can be introduced before, during or after introduction of the recombinant vector. For instance, the plasmid can be co-infected into the culture along with the helper virus. The cells are then cultured for a suitable period, typically 2-5 days, in conditions suitable for replication and packaging as known in the art (see references above and examples below). Lysates are prepared, and the recombinant AAV vector 35 40 particles are purified by techniques known in the art. Alternatively, helper virus functions are provided to the cell on recombinant vectors, such as plasmids.

Purification of recombinant vectors

45 Recombinant vectors encapsidated in AAV particles prepared using the methods and compositions of the present invention can be purified according to techniques known in the art, see, e.g., the various AAV references cited above. Alternatively, improved 30

5 purification techniques can be employed, such as those described by Atkinson et al. in
International Patent Application No. PCT/US98/18600.

10 **Introduction of heterologous genes into a cell using encapsidated recombinant vectors
of the invention**

15 5 The recombinant vectors encapsidated into AAV particles can be used to deliver
polynucleotides to target cells either *in vitro*, *in vivo*, or *ex vivo*, as described in the
references cited herein and in the art. For delivery *in vivo*, the recombinant vectors
20 encapsidated in AAV particles will typically be contained in a physiological suitable
buffered solution that can optionally comprise one or more components that promote
sterility, stability and/or activity. Any means convenient for introducing the vector
25 preparation to a desired location within the body can be employed, including, for example,
by intravenous or localized injection, by infusion from a catheter or by aerosol delivery.

30 The examples presented below are provided as a further guide to a practitioner of
ordinary skill in the art, and are not meant to be limiting in any way.

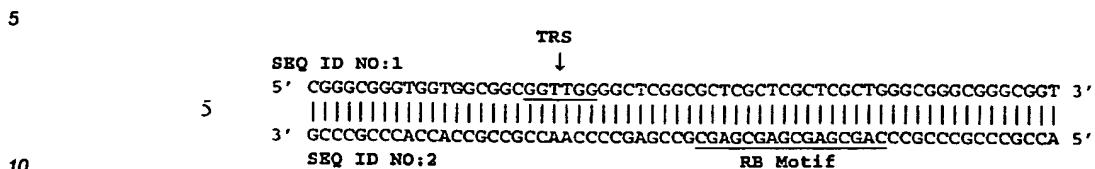
35 15 **EXAMPLES**

40 **EXAMPLE 1**

45 *Construction of recombinant vectors comprising a non-AAV ITR encapsidation element
operably linked to a heterologous gene, and cells comprising the vectors.*

50 20 1. Construction of a recombinant vector employing P1 as an exemplary encapsidation
element

55 25 An exemplary P1 sequence we used as the source of encapsidation element
comprises nucleotides 354-468 of the AAV S1 locus (Kelman et al (1994) Curr. Opin.
Genet. Dev. 4:185-195; Weitzman et al (1994) Proc. Natl. Acad. Sci. 91:5808-5817).
30 40 Shown below is the nucleotide sequence of a P1 encapsidation element (SEQ ID NOs. 1
and 2), including a presumed terminal resolution site (TRS) at nucleotides 19-24 of SEQ ID
NO:1 (i.e., nucleotides 372-377 of the AAV S1 locus), and a presumed Rep binding motif
(RB Motif, also known as a Rep-binding site or RBS), at nucleotides 33-48 of SEQ ID
45 30 NO:1 (i.e., nucleotides 386-401 of the AAV S1 locus). Also indicated (by the downward-
pointing arrow) is the presumed Rep cleavage site located between the thymidines of the
TRS.



2. Construction of p5repCap

10 We linked a P1 element (as described above) to AAV *rep* and *cap* genes that remained operably linked to their native AAV promoters. As a first step in that process, an AAV packaging cassette, p5repCap, comprising the AAV *rep* and *cap* encoding sequences transcriptionally linked to the native p5, p19 and p40 promoters and followed by the AAV2 polyadenylation signal, was constructed as follows. Briefly, a fragment from pAV2

15 comprising nucleotides 193 to 379 (Srivastava et al. (1983) J. Virol. 45:555-564) was obtained by PCR amplification. The design of the PCR primers resulted in addition of a BglII site at the 5' end of the amplified fragment and encompassed the PpuMI site (at

20 AAV-2 nucleotide 350) close to the 3' end. The PCR-amplified DNA was digested with BglII and PpuMI to generate a restriction fragment comprising AAV-2 nucleotides 193-350. A restriction fragment comprising nucleotides 351-4498 of pAV2 was isolated from

25 pAV2 by digestion with PpuMI and SnaBI. These two fragments (representing nucleotides 193-4498 of pAV2) were ligated into a tgLS(+)HyTK retroviral vector (S.D. Lupton et al., Molecular and Cellular Biology, 11: 3374-3378, 1991) in a four-way ligation that also included a StuI-BstEII fragment of tgLS(+)HyTK and a BstEII-StuI fragment of

30 tgLS(+)HyTK to which a BglII linker had been attached at the StuI end. This ligation generated tgLS(+)HyTK-repCap. Subsequently, a BglII - Clal fragment from tgLS(+)HyTK-repCap, including AAV *rep* and *cap* genes transcriptionally linked to the native p5, p19 and p40 promoters and followed by the AAV2 polyadenylation signal, was

35 isolated and cloned into the BamHI and Clal sites of pSP72 (Promega).

40

30

3. Construction of p5repCapDHFR

45 Expression plasmid p5repCapDHFR was constructed for the purpose of producing an integrated packaging line including the construct p5repCap (Example 1, section 2) and a modified dihydrofolate reductase gene (DHFR) as a selectable marker. Specifically,

5 p5repcap (Example 1, section 2) was linearized at a PvuII site located just upstream of the
rep gene, and blunt-end ligated to a 1.8 kb fragment of pFR400 (Simonsen et al. (1983)
10 Proc. Natl. Acad. Sci. USA 80:2495-2499). This pFR400 fragment comprises a modified
DHFR gene, with a reduced affinity for methotrexate (Mtx), transcriptionally linked to the
15 SV40 early promoter and followed by the polyadenylation site from the Hepatitis B virus
(HBV) surface antigen gene. The pFR400 fragment was prepared by first digesting with
Sall, followed by a four base pair fill-in (to generate a blunt end) and subsequent PvuII
digestion and gel purification. The resulting construct, p5repcapDHFR (Figure 4), contains
20 a DHFR gene whose transcription is regulated by an upstream SV40 early promoter and a
downstream Hepatitis B Virus polyadenylation site. Immediately downstream of this
DHFR transcriptional cassette lie the AAV rep and cap genes, followed by an AAV
polyadenylation site.

4. Addition of P1 to a repcap-containing plasmid: Construction of P1RCD

25 15 A P1 element (Example 1, section 1) was then incorporated into expression plasmid
p5repcapDHFR (Example 1, section 3). In the construction of the plasmid, "P1RCD",
30 containing this packaging cassette, the P1 element was inserted downstream of the AAV
polyadenylation signal in p5repcapDHFR in an orientation such that replication initiating
from the P1 element proceeds first into the cap gene and then into the rep gene (i.e.,
20 replication initiates at the 3' -OH of the TRS on the anti-sense strand and proceeds in a 5'-
to-3' direction towards the cap gene). To facilitate insertion of the P1 element into
35 p5repcapDHFR, a pair of oligonucleotides was synthesized which include the P1 sequence
flanked by ends compatible with a BglII restriction site (see sequences below, SEQ ID
NOs. 3 and 4). The pair was annealed, then ligated to p5repcapDHFR previously
25 linearized at a BglII site located just downstream of the AAV polyadenylation site
(Example 1, section 3, nucleotide 6217). A clone named P1RCD was selected, containing
40 a P1 insert in an orientation such that replication initiated at P1 proceeds in the direction of
the cap and rep genes (Figure 5). This vector contains no AAV ITR sequences.

45 30 P1 Oligonucleotide pair:

SEQ ID NO:3 RB Motif
5' GATCACTAGTACCCCCCCCCCGCCACCGAGGGAGCGCGAGGCCCAACCGCCGCCACCCACCCGGGA 3'
3' TGATCATGGGGGGGGGGGGCTGCTCGCTCGCTCGGGCTGGGGTTGGGGGGGGGGGGCTCTAGA 5'

5

SEQ ID NO:4

TRS

5. Construction of rAAV vector ACAPSN

The plasmid ACAPSN was constructed according to Lynch et al. (1997) *Circ. Res.* 80: 497-505 and PCT Publication WO 97/32990, as follows. The ITR sequences and plasmid backbone were derived from AAV-CFTR. Afione et al. (1996) *J. Virol.* 70:3235-3241. Briefly, the AAV-CFTR vector was digested with Xhol and SnaBI and the ITRs and plasmid backbone were gel isolated. An Xhol to SnaBI fragment containing a portion of the CMV promoter (nucleotides -671 to -464) [See, e.g., Boshart, et al., *Cell*, 41: 521-530 (1985)] was gel isolated and ligated to the ITR plasmid backbone fragment derived from AAV-CFTR to generate "pAAV-CMV (SnaBI)." Next, an Spel to SnaBI fragment containing the synthetic polyadenylation signal was inserted into Spel/SnaBI digested pAAV-CMV (SnaB1) to generate "pAAV-CMV (Spel)-spA." The pAAV-CMV (Spel)-spA vector contains nucleotides -671 to -584 of the CMV promoter. Next, the human placental alkaline phosphatase cDNA sequence linked to the Simian virus 40 promoter driving the *E. coli* neomycin gene was isolated from LAPS [See, e.g., Clowes et al., 1994, *J. Clin. Invest.* 93:644-651] as an Spel to NheI fragment and inserted into pAAV-CMV (Spel)-spA (which had been linearized with Spel) to create "pAAV-APSN." An Spel to NheI fragment containing CMV promoter nucleotides -585 to + 71 was inserted into Spel-linearized pAAV-APSN to generate vector "ACAPSN."

6. Production of packaging cell lines containing P1RCD

35 Polyclonal cell lines with an integrated AAV packaging cassette containing the P1 element (P1RCD) were produced by electroporation of HeLa cells. Specifically, 4×10^6 HeLa cells were electroporated with 12 μ g DNA (P1RCD) that had been linearized with 25 PvuII restriction endonuclease, which cleaves just upstream of the SV40 promoter-DHFR 40 gene cassette. The cells were electroporated in serum free DMEM using a BioRad Gene Pulser at 0.25 Volts and 960 μ F. After electroporation, cells were resuspended in 45 Dulbecco's Modified Eagles medium, 10% fetal bovine serum, with 1% penicillin and 30 streptomycin (DMEM complete) and allowed to recover at 37°C in a humidified atmosphere of 10% CO₂. After 24 hours, cells were subjected to selection in complete medium containing 500 nM methotrexate.

5

7. Production of P1RCD clonal cell lines

10 P1RCD polyclonal cells were plated in 96-well plates at a density of 1, 0.3, and 0.1
5 cell per well in DMEM containing 10 % dialyzed fetal bovine serum, 1% penicillin,
streptomycin, and L-glutamine plus 500 nM methotrexate. Wells were visually inspected
15 for cell growth and the presence of single colonies. Clones were expanded from 96-well
plates with 15 or fewer positive wells per plate and from wells containing single colonies.
Cells were maintained under selection of 500 nM methotrexate in DMEM containing 10%
20 dialyzed serum until individual clones were frozen. Clones were screened for the presence
of the P1RCD construct. Positive clonal cell lines were frozen and stored in liquid
nitrogen. The C29 clonal cell line containing the P1RCD construct was chosen for
subsequent experiments.

25 15 8. Production of producer cell lines P1/ACAPSN and P1/ALinBg

30 Producer cell line P1/ACAPSN was generated by electroporating P1RCD C29
packaging cells in an analogous manner as the P1RCD packaging line above. Specifically,
4 x 10⁶ P1RCD C29 cells were electroporated with 10µg of tgACAPSN DNA that had been
35 linearized with Xmn I endonuclease. Electroporation conditions are described in Example
1, section 6. After electroporation, the cells were resuspended in DMEM complete and
20 allowed to recover at 37°C for 24 hours. Cells were then subjected to selection in complete
media containing 1 mg/ml G418. Clones of P1/ACAPSN were selected and expanded in
35 the manner described above (Example 1, section 7) using 1 mg/ml G418 as selection
media. The P1/ACAPSN C19 cell line was chosen for subsequent experiments. Clones
25 were screened for the ability to produce ACAPSN virions according to Example 2.

40 P1/ALinBg clones were produced in an analogous manner by electroporating
P1RCD C29 cells with ALinBg DNA.

45 EXAMPLE 2

30 *P1 element promotes encapsidation of operably linked gene into AAV particles.*

5 1. Production of virions

10 C29 cells (Example 1, section 7) were seeded at a density of 5×10^6 cells in a T225
 5 cm² flask one day prior to infection with adenovirus (Ad). Four replicate flasks were
 seeded. Twenty-four hours later, one flask of cells was treated with trypsin and the number
 15 of cells counted. The remaining three flasks of cells were infected with Ad at a multiplicity
 20 of infection of 10. Seventy-two hours later cells were collected by centrifugation and
 resuspended to a concentration of 5×10^6 cells/mL in 50 mM TRIS, pH 8.0, 5 mM MgCl₂,
 1 mM EDTA, 5% glycerol (TMEG). Cells were subjected to repeated freeze/thaw (-
 70°C/37°C) cycles and sonication (4 x 15 sec bursts). After confirmation that greater than
 95% of the cells were lysed, cell debris was removed by low speed centrifugation. The
 resulting cleared lysates were examined for the presence of encapsidated P1RCD DNA
 sequences.

25 2. DRP slot-blot analysis

30 Encapsidated DNA sequences were examined by DNA hybridization following
 35 DNase treatment of cleared lysates. A number of radiolabeled probes were generated
 40 which spanned the P1RCD construct: cap; rep-cap; DHFR#1 (DHFR gene and hepatitis B
 polyadenylation signal); and DHFR #2 (DHFR coding sequences only). The number of
 DNase Resistant Particles (DRP) was quantitated by comparison to a standard curve
 included on each slot-blot. P1RCD plasmid DNA was used to generate standards.

45 DNase resistant, i.e. encapsidated, DNA sequences were detected in cleared lysates
 generated from C29 cells with each of the P1RCD probes, as shown in Table 1, below. In
 general, the number of DNase Resistant Particles was on the order of 1×10^{10} /mL. This
 level of encapsidation is comparable to that typically seen with ITR-mediated packaging of
 AAV vector genomes.

50 Table 1

Probe	DRP/mL
rep-cap	1×10^{10}
cap	1×10^{10}
DHFR #1	1.3×10^{10}
DHFR #2	1.3×10^{10}

5

EXAMPLE 3.*Characterization of the P1 encapsidation element.***1. The P1 encapsidation element is included in the encapsidated DNA**

10

DNase resistant, i.e. encapsidated, DNA sequences were detected in cleared lysates generated from C29 cells using a P1 probe. Oligonucleotides comprising the P1 element were synthesized, annealed and end-labeled. Similar numbers of virions were detected with the P1 probe (2×10^{10} DRP/mL) as previously detected with the rep-cap, cap and DHFR probes. This indicates that the P1 encapsidation element is included in the encapsidated DNA sequences.

15

10 2. P1 encapsidation element promotes encapsidation of sense and anti-sense DNA strands at an equal ratio.

20

Duplicate slot-blots of DNase-treated C29 cleared lysate were individually hybridized with oligonucleotide probes representing the 5' to 3' and 3' to 5' sequences of the P1 element. Titers of DRPs observed with the sense and anti-sense P1 probes were 2.6×10^{10} and 1.3×10^{10} DRP/mL, respectively. It appears that the P1 encapsidation element directs encapsidation of DNA strands of either polarity at equal frequency.

25

30 3. P1 encapsidation element promotes packaging in a vector producer cell line in the presence of ITR sequences

35

Slot-blots of DNase treated P1/ACAPSN C19 cleared lysate were hybridized with the cap, DHFR#1 and DHFR#2 probes described in Example 2, section 2, above. The number of ACAPSN vector particles present was also determined using a CMV probe. The results are shown in Table 2. "NA" indicates "not applicable"

Table 2

Probe	DRP/mL (P1 packaging)	DRP/mL (ITR Packaging)
cap	3×10^9	NA
DHFR #1	2.7×10^9	NA
DHFR #2	2.3×10^9	NA
CMV	NA	1.3×10^{11}

40 25 Both ITR- and P1-promoted encapsidation of DNA sequences were observed in P1/ACAPSN C19 cleared lysate. The titer of particles containing recombinant polynucleotides operably linked to P1 (i.e., P1-directed encapsidation) was one-half log

45

5 lower than previously observed in the C29 clonal cell line, which lacks an ITR-flanked
ACAPSN vector cassette. These data demonstrate that the P1 element can function as a
packaging signal even in the presence of a *bona fida* AAV ITR packaging signal.

10 5 4. Encapsidated DNA sequences in purified recombinant vector preparations from HeLa
cells containing a P1 element

15 Large-scale vector preparations were manufactured from the P1/ACAPSN C19 cell
line and purified by CsCl ultra-centrifugation and ion-exchange chromatography. Two
independent lots of vector were manufactured. In addition to the ACAPSN vector
particles, the purified preparations contained encapsidated DNase resistant particles which
10 contained recombinant polynucleotides operably linked to P1.

20 Another P1 producer cell line was independently generated from an AAV vector
carrying the β -galactosidase reporter gene (ALinBg). Vector preparations manufactured
from the P1/ALinBg producer cell line also contained DNase resistant particles containing
recombinant polynucleotides operably linked to P1, in addition to ALinBg vector particles.

25 15 Southern Analysis

30 The P1-encapsidated DNA was examined by Southern blot analysis. Purified
virions from the P1/ACAPSN and P1/ALiNBg cell lines were lysed and the encapsidated
DNA fractionated by electrophoresis in alkaline gels. A predominant band of
35 approximately 4.7 kb in size was observed in all vector lots when hybridized with a rep-cap
20 probe, as shown in Figure 6. This suggests that the predominant DNA species packaged
using the P1 packaging signal are similar in size to the wild-type AAV genome length, i.e
the normal AAV packaging capacity.

40 25 Thus, using two different AAV vectors and two producer cell lines independently
derived from the C29 packaging cell line, we have observed P1 promoted encapsidation of
45 30 cis linked sequences. Furthermore, P1-promoted packaging occurred in the presence of
ITR-mediated encapsidation of recombinant AAV vectors. The P1 packaged sequences
were co-purified with rAAV virions by CsCl isopycnic ultra-centrifugation and survived
treatment with DNase and heating to 54 °C for 10 minutes. This indicates that P1 promotes
encapsidation into AAV particles that are robust and can be purified by methods used for
recombinant AAV vectors.

5

EXAMPLE 4.

Construction and encapsidation of a recombinant polynucleotide comprising a P1 element operably linked to coding sequences for CFTR.

10

5 The region comprising AAV rep and cap genes is excised by BgIII restriction endonuclease digestion from P1RCD and the fragment including P1 element and DHFR gene is isolated. A DNA fragment encoding CFTR and having compatible restriction endonuclease overhangs with the P1-containing fragment is isolated. The P1-containing fragment is ligated to the DNA fragment encoding CFTR, to produce a recombinant polynucleotide in which a P1 element is operably linked to sequences encoding CFTR.

15

10 This recombinant polynucleotide is introduced into a mammalian cell line producing AAV rep and cap gene products, and subsequently the cell line is infected with Ad helper virus.

20

25 Cells are lysed and DRPs are measured in the cleared lysates, as described above, then analyzed by slot blot hybridization with probes which hybridize to the P1 element and to CFTR-coding regions.

35

30 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, 20 which is delineated by the appended claims.

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Claims

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CLAIMS

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1. A method for producing a recombinant polynucleotide encapsidated in an adeno-associated virus (AAV) particle, comprising providing a mammalian cell which produces AAV rep and cap gene products, wherein said mammalian cell contains the recombinant polynucleotide which comprises a heterologous gene operably linked to an encapsidation element which promotes encapsidation of said heterologous gene into an AAV particle, and wherein said encapsidation element is other than an AAV inverted terminal repeat (ITR) or its D sequence.

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2. The method of claim 1, wherein said encapsidation element has a nucleotide sequence which has at least about 30 % nucleotide sequence identity with SEQ ID NO:1.

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3. The method of claim 1, wherein said encapsidation element has a nucleotide sequence which has at least about 47 % nucleotide sequence identity with SEQ ID NO:1.

30

4. The method of claim 1, wherein said encapsidation element comprises at least about 35 contiguous nucleotides of the nucleotide sequence depicted in SEQ ID NO:1.

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5. The method of claim 1, wherein said encapsidation element comprises a terminal resolution site.

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6. The method of claim 1, wherein said encapsidation element comprises a binding site for AAV Rep68 and Rep78 proteins.

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7. The method of claim 1, wherein said encapsidation element comprises a terminal resolution site and a binding site for AAV Rep68 and Rep78 proteins.

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8. The method of claim 1, wherein said encapsidation element comprises the nucleotide sequence GGTTGG(X)_nGCXCGCTCGCTGCTX, wherein X is any nucleotide and n is an integer from 1 to about 20.

5 9. The method of claim 1, wherein said encapsidation element comprises a
nucleotide sequence having the sequence of nucleotides 19 to 48 of SEQ ID NO:1.

10 10. The method of claim 1, wherein said encapsidation element has the nucleotide
5 sequence depicted in SEQ ID NO:1.

15 11. The method of claim 1, wherein the encapsidation activity of said encapsidation
element is activated by helper function.

20 10 12. The method of claim 11, wherein said helper function is provided by an
adenovirus.

25 15 13. The method of claim 1, wherein said AAV rep and cap gene products produced
by said mammalian cell are encoded by AAV rep and cap genes which are stably integrated
15 into the genome of said cell.

30 30 14. The method of claim 1, wherein said AAV rep and cap gene products are
encoded by an extrachromosomal polynucleotide.

35 20 15. An isolated recombinant polynucleotide sequence comprising a heterologous
gene flanked by encapsidation elements other than adeno-associated virus (AAV) inverted
35 terminal repeats (ITR) or AAV ITR D sequences, wherein said encapsidation elements
promote encapsidation of said heterologous gene in the presence of AAV rep and cap gene
products and helper virus function.

40 25 16. The isolated recombinant polynucleotide of claim 15, wherein said
encapsidation element has a nucleotide sequence which has at least about 30 % nucleotide
sequence identity with SEQ ID NO:1.

45 30 17. The isolated recombinant polynucleotide of claim 15, wherein said
encapsidation element has a nucleotide sequence which has at least about 47 % nucleotide
sequence identity with SEQ ID NO:1.

5

18. The isolated recombinant polynucleotide of claim 15, wherein said encapsidation element comprises at least about 35 contiguous nucleotides of the nucleotide sequence depicted in SEQ ID NO:1.

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19. The isolated recombinant polynucleotide of claim 15, wherein said encapsidation element comprises a terminal resolution site.

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20. The isolated recombinant polynucleotide of claim 15, wherein said

encapsidation element comprises a binding site for AAV Rep68 and Rep78 proteins.

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21. The isolated recombinant polynucleotide of claim 15, wherein said encapsidation element comprises a terminal resolution site and a binding site for AAV Rep68 and Rep78 proteins.

15

22. The isolated recombinant polynucleotide of claim 15, wherein said encapsidation element comprises the nucleotide sequence

GGTTGG(X)nGCXCGCTCGCTGCTX, wherein X is any nucleotide and n is an integer from 1 to about 20.

30

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23. The isolated recombinant polynucleotide of claim 15, wherein said

encapsidation element comprises a nucleotide sequence having the sequence of nucleotides 19 to 48 of SEQ ID NO:1.

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24. The isolated recombinant polynucleotide of claim 15, wherein said encapsidation element has the nucleotide sequence depicted in SEQ ID NO:1.

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25. The isolated recombinant polynucleotide of claim 15, wherein the encapsidation activity of said encapsidation element is activated by helper function.

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26. The isolated recombinant polynucleotide of claim 15, wherein said helper function is provided by an adenovirus.

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27. The isolated recombinant polynucleotide of claim 15, wherein said AAV rep and cap gene products produced by said mammalian cell are encoded by AAV rep and cap genes which are stably integrated into the genome of said cell.

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28. The isolated recombinant polynucleotide of claim 15, wherein said AAV rep and cap gene products are encoded by an extrachromosomal polynucleotide.

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29. The isolated recombinant polynucleotide of claim 15, wherein said heterologous gene encodes a functional CFTR polypeptide.

20

30. A method for generating a packaging cell capable of producing stocks of a recombinant polynucleotide comprising a heterologous gene encapsidated in an adeno-associated virus (AAV) particle, comprising transfecting mammalian cells which produce AAV rep and cap gene products with the recombinant polynucleotide, wherein said recombinant polynucleotide comprises a heterologous gene operably linked to an encapsidation element other than an AAV inverted terminal repeat (ITR) or an AAV ITR D sequence, wherein said encapsidation element promotes encapsidation of said heterologous gene into an AAV particle.

25

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31. The method of claim 30, wherein said AAV rep and cap gene products produced by said mammalian cell are encoded by AAV rep and cap genes stably integrated into the genome of the cell.

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32. The method of claim 30, wherein said AAV rep and cap gene products are encoded by an extrachromosomal polynucleotide.

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33. The method of claim 30, wherein said recombinant polynucleotide further comprises a selectable marker.

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34. The method of claim 30, wherein said recombinant polynucleotide integrates into the genome of the cell.

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35. A packaging cell produced by the method of claim 18.

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36. A mammalian packaging cell for producing stocks of a recombinant
5 polynucleotide encapsidated in an adeno-associated virus (AAV) particle, wherein said
packaging cell comprises the recombinant polynucleotide, wherein said cell produces
15 adeno-associated virus (AAV) rep and cap gene products, and wherein said recombinant
polynucleotide comprises a heterologous gene operably linked to an encapsidation element
other than an AAV inverted terminal repeat (ITR) or an AAV ITR D sequence, wherein
10 said encapsidation element promotes encapsidation of said heterologous gene.

20

37. The mammalian packaging cell of claim 36, wherein said rep and said cap gene
products are encoded by genes stably integrated into the genome of the cell.

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38. The mammalian packaging cell of claim 36, wherein said rep and said cap gene
products are encoded by extrachromosomal genes.

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39. The mammalian packaging cell of claim 36, wherein said recombinant vector is
maintained episomally.

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FIGURE 1

CCCCGGGCGGGCGGGCGGGCGGGTGGTGGCGGGGTTGGGGCTCGGGCGCTCGCTCGCTCGCTGGGCGGGC
GGGCGGTGCGATGTCGGAGAGGATGGCCGGCGGCTGGCCCCGG

FIGURE 2

FIGURE 3

P1
 5' CGGGCGGGTGGTGGCGCGCGTTGGGGCTCGGC-----
 GCTCGCTCGCTCGCTGGCGGGCGGGCGGT
 5' TTACCCCTAGTGATTGGAGTTGCCACTCCCTCTGCGCGCTCGCTCGCTCGGTGGGC
AAV1 ITR

P1
 5' CGGGCGGGTGGTGGCGCGCGTTGGGGCTCGGC-----
 GCTCGCTCGCTCGCTGGCGGGCGGGCGGT
 5' GCCATACCTCTAGTGATTGGAGTTGCCACTCCCTATGCGCACTCGCTCGCTCGGTGGGC
AAV3 ITR

P1
 5' CGGGCGGGTGGTGGCGCGCGTTGGGGCTCGGC-----
 GCTCGCTCGCTCGCTGGCGGGCGGGCGGT
 5' GGGAAACCTAGATGATTGGAGTTGCCACTCCCTATGCGCGCTCGCTCACTCGGCC
AAV4 ITR

P1
 5' CGGGCGGGTGGTGGCGCGCGTTGGGGCTCGGC-----
 GCTCGCTCGCTCGCTGGCGGGCGGGCGGT
 5' ACAAACCTCCTGCTTGAGAGGTGGCACTCTCCCCCTGTCGCGTTCGCTCGCTGGCTCGTTGGGGGG
AAV5 ITR

P1
 5' CGGGCGGGTGGTGGCGCGCGTTGGGGCTCGGC-----
 GCTCGCTCGCTCGCTGGCGGGCGGGCGGT
 5' TTACCCCTAGTGATTGGAGTTGCCACTCCCTCTGCGCGCTCGCTCGCTCACTGAGGCC
AAV6 ITR

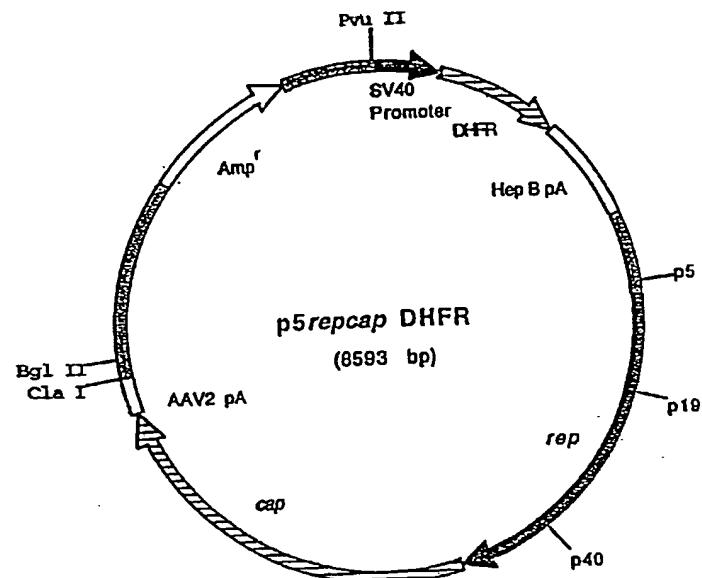
Figure 4

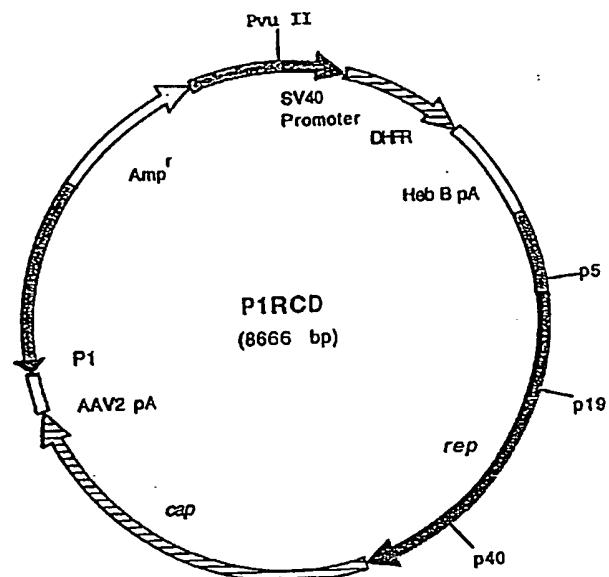
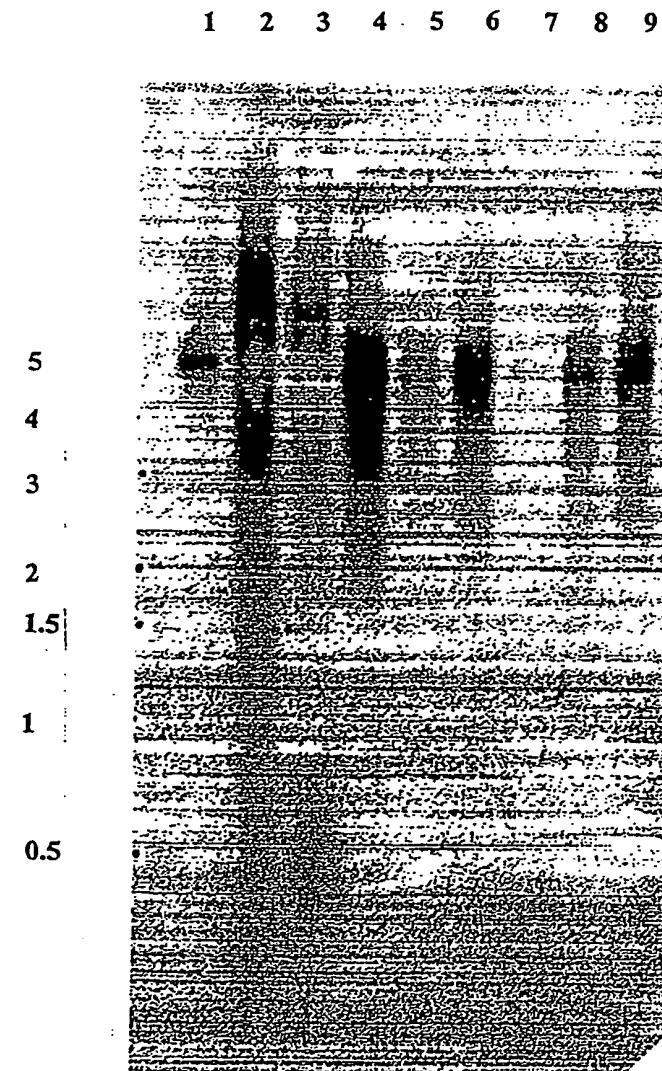
Figure 5

Figure 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/04755

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